Bioinspired, nanoscale approaches in contemporary bioanalytics (Review)

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The genesis for this topical review stems from the interdisciplinary Biointerfaces International conference 2016 (BI 2016) in Zurich, Switzerland, wherein the need for advances in analytical tools was both expressed and addressed. Pushing the limits of detection for characterizing individual components, such as single proteins, single drug-delivery vehicles, or probing single living cells in a more natural environment, will contribute to the understanding of the complex biomolecular systems central to a number of applications including medical diagnostics, tissue engineering, and drug screening and delivery. Accordingly, the authors begin with an overview of single nanoparticle analytics highlighting two emerging techniques and how they compare with existing techniques. The first is based on single particle tracking of nanoparticles tethered to a mobile supported lipid bilayer, enabling the simultaneous characterization of both size and composition of individual nanoparticles. The second technique is based on probing variations in the ionic conduction across nanoscale apertures for detection of not only nanoparticles but also membrane-tethered proteins, thereby allowing a multiparameter characterization of individual nanoscopic objects, addressing their size, shape, charge, and dipole moment. Subsequently, the authors lead into an example of an area of application that stands to benefit from such advances in bioanalytics, namely, the development of biomimetic lipid- and polymer-based assemblies as stimuli-responsive artificial organelles and nanocarriers designed to optimize delivery of next generation high-molecular-weight biological drugs. This in turn motivates the need for additional advanced techniques for investigating the cellular response to drug delivery, and so the review returns again to bioanalytics, in this case single-cell analysis, while highlighting a technique capable of probing and manipulating the content of individual living cells via fluidic force microscopy. In presenting a concerted movement in the field of bioinspired bioanalytics, positioned in the context of drug delivery, while also noting the critical role of surface modifications, it is the authors' aim to evaluate progress in the field of single component bioanalytics and to emphasize the impact of initiating and maintaining a fruitful dialogue among scientists, together with clinicians and industry, to guide future directions in this area and to steer innovation to successful translation. https://doi.org/10.1116/1.5037582

I. INTRODUCTION

The interface between engineered materials and biological systems is one of the most rapidly expanding areas of science and technology and now encompasses medical implants, drug delivery, regenerative medicine, and medical diagnostics. Future progress depends strongly on the development of new bioanalytical tools to understand these interfaces and to develop a functional understanding of complex biomolecular systems. The capability to both characterize and quantify single nanoparticles in solution or single proteins in their native conformation, without the need for labeling, is of great interest and is expected to contribute to a number of applications ranging from identifying and sorting optimal drug-delivery vehicles to rapid and sensitive protein detection from a complex biological medium. As an illustrative example, next generation drug-delivery vehicles

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carrying biologicals, such as proteins or nucleic acids, are often designed today based on information that has been garnered through existing analytical techniques about how natural biological nanoparticles, such as viruses or exosomes, transfer genetic information between cells *in vivo*, including characteristics such as remaining stable against biological degradation, interacting weakly with serum proteins and (nontarget) cells, reaching their target tissues with high efficiency, and even entering into target cells to induce the desired functional response. When considering the complexity of steps that must be precisely understood and controlled for the development of next generation functional drug-delivery vehicles—as just one example out of a number of biomedical applications—it is clear that future progress depends critically upon the development of bioanalytical tools capable of studying single nanoparticles and biomolecular complexes, individually, in their native environments. Furthermore, the ability to subsequently investigate cellular uptake and the ensuing effects on individual cells presents as yet another area of bioanalytics for which a driver toward higher precision and physiologically relevant *in vitro* cell analysis persists.[1–3]

Correspondingly, this topical review will focus on recent advances of precisely such bioanalytical tools that make use of and contribute to our understanding of the supramolecular assemblies found in nature to achieve new levels of detection in the characterization of single proteins, single nanocarriers, and in our ability to interact with single living cells. The interdisciplinary nature of this paper is in line with, and inspired by, the inaugural Biointerfaces International (BI) conference 2016 in Zurich, Switzerland, that aimed precisely at fostering cross-disciplinary dialogues spanning from academic laboratories to hospital clinics, as well as to industry. The interested reader is encouraged to seek out the interview, in the Appendix, of Dr. Marcus Textor, the chairman and visionary behind the inaugural Biointerface International 2016, that further illustrates the rationale and need for this type of open communication. Dr. Textor outlines the importance of an ever wider interdisciplinary approach to creating biologically functional materials for both diagnostic applications, e.g., drug screening, toxicity, disease monitoring, as well as for applications in targeted drug delivery and regenerative medicine. While expressing his excitement for bioinspired solutions, Dr. Textor further emphasizes the importance of tackling the hurdles for translation of advanced technology and the need for improved bioanalytical tools in enhancing both our understanding of and the further advancements of functional materials in the medtech field.

Following this vision, an overview of the field of single nanoparticle analytics is described as the starting point, in Sec. II, highlighting a new technique based on single particle tracking (SPT) of membrane-tethered nanoparticles enabling the characterization of both the size and content of single biological nanoparticles.[4] It stands as a detailed example of the field, which together with a review of alternative tools used in single nanoparticle analytics, underscores the importance of addressing nanoparticle heterogeneity with respect to size and molecular content. In Sec. III, the focus turns to a complementary approach based on probing variations in the ionic conduction across nanoscale apertures that further enables not only the detection of nanoscopic entities down to individual protein molecules but also makes it possible to characterize them with respect to, for example, their size, shape, charge, and dipole moment,[5] thus complementing the method presented in Sec. II. While highly relevant from both a purely fundamental and applied diagnostic perspective, the possibility to characterize the physicochemical properties of individual nanoparticles and proteins will also aid in our understanding of how dynamic engagement of multiple weak intermolecular interactions could be optimized in the assembly of supramolecular systems, as in drug-delivery vehicles, for example. Therefore, in Sec. IV, drug-delivery systems are discussed as an example of a field that stands to benefit from the types of bioanalytical techniques presented in Secs. II and III, with a focus on the development of biomimetic lipid- and polymer-based assemblies designed to optimize delivery of next generation high-molecular-weight biological drugs or to generate artificial organelles able to act, *in vivo*, as cellular implants.[6] Furthermore, the successful translation to clinical use of these advanced drug-delivery systems and artificial organelles will depend on extensive evaluations, thus leading us into Sec. V wherein the most promising analytical tools for single-cell analyses (SCA) are presented with a particular focus on a technique based on probing and manipulating the content of an individual cell through fluidic force microscopy.[7] We close the paper with an outlook of the most crucial bottleneck issues involved in quantitative nanoparticle analysis, with particular emphasis on the importance of sophisticated surface modifications for these tools to function properly, and the need not only to analyze heterogeneous samples with single nanoparticle resolution but also to sort and enrich with the same single molecule precision. Furthermore, a perspective on the obstacles to commercialization is presented along with a discussion of the need for biologically designed tissue engineered organoid model systems, combined with suitable analytical tools, to act as *in vitro* disease models for studying disease biology, drug development, and human toxicity.

II. SINGLE NANOPARTICLE ANALYTICS AND 2D FLOW NANOMETRY

Molecular self-assembly, governed by weak intermolecular interactions, has evolved as a central design principle of small-scale supramolecular assemblies, including viruses and membrane-enveloped biological nanoparticles, such as exosomes, that participate in intercellular communication.[8] Obviously, the design of biomimetic drug-delivery nanocarriers, as schematically illustrated in Fig. 1, is aimed at performing similar tasks to that of exosomes or viruses and is therefore very demanding as it requires exact control of multiple precisely fine-tuned supramolecular self-assembly processes.[6,9,10] The magnitude of this challenge becomes ever

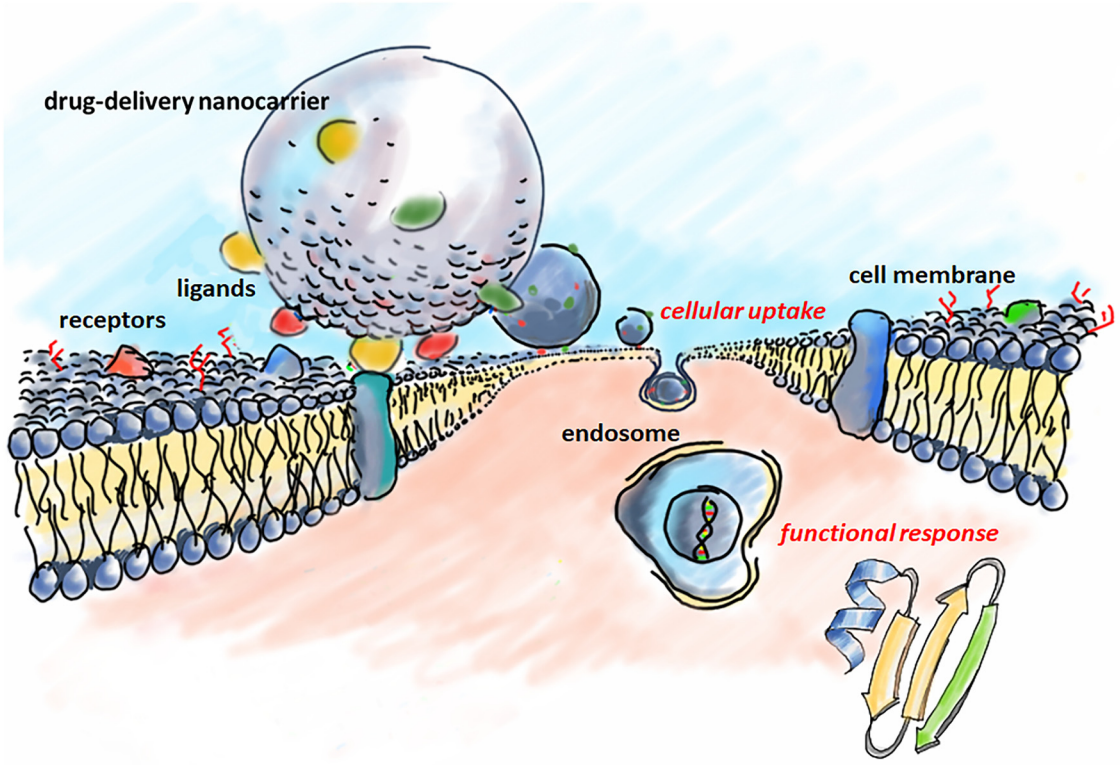

FIG. 1. Schematic illustration of nanoparticle binding to a cell membrane utilizing different ligands directed to different cell-membrane receptors, followed by uptake, endosomal release, and a functional response.

more clear by considering that, despite significant research efforts, the interdependent roles of particle dimension, structure, shape, and flexibility with respect to the type of binder, density, and accompanied multivalent binding for efficient cellular uptake and cellular response still remain largely unknown.[11] For example, both cellular uptake and functional response of cells targeted by biological nanoparticles are known to depend on nanoparticle size, ligand density,[12] shape,[13] and molecular composition, which in turn depend on the physicochemical properties of the individual components and their relative concentration as well as preparation method.[3,14–16] Progress in this field is thus strongly dependent on developing the ability to characterize the exact physicochemical properties of both natural and artificially produced bioinspired nanocarriers.

Methods conventionally used to characterize soft biological nanoparticles can be divided according to the nature of the information they provide. Depending on the application, it is also important to consider whether they provide single nanoparticle or ensemble-averaging information. In Table I, the most commonly used nanoparticle characterization methods, with single nanoparticle resolution, are categorized according to the resolution they offer, with respect to the smallest detectable size, together with a rough indication regarding the required concentration and total sample volume needed to reach sufficient resolution and statistics. Here, we will focus on methods offering single nanoparticle resolution; however, we acknowledge the value of ensemble-averaging methods, such as dynamic light scattering (DLS)[17] and neutron and x-ray scattering,[18] when used to analyze molecularly homogeneous samples with narrow size distributions, in which case the physical and structural information provided is often superior compared with that offered by methods with single nanoparticle sensitivity. For example, DLS can in such cases offer not only precise size determinations but also accurate zeta-potential and concentration determinations.[19] Neutron and x-ray scattering techniques, in contrast, offer very detailed structural information, as, for example, recently illustrated by Lindfors *et al.*, when showing that successful mRNA delivery is critically dependent not only on nanoparticle size but also on structure.[20] Further, ensemble-averaging surface analytical tools, such as quartz crystal microbalance, ellipsometry, and surface plasmon resonance (SPR), can provide an accurate determination of the nanoparticle size by quantifying the thickness of films made up of nanoparticles.[21,22] In particular, by modifying the surface with antibody binders, even heterogeneous biological nanoparticles can be analyzed via selective binding of subpopulations characterized by carrying certain biological markers,[23] and the concentration can also be determined from the rate of binding.[22] In many situations, however, the heterogeneous nature of nanoparticles severely limits the applicability of ensemble-averaging methods. For example, although it has been known for more than a decade that extracellular vesicles (EVs) can communicate genetic information between cells,[24] it was not until recently that it has become clear that it is only a very minute fraction of all cell-produced EVs that has the size, molecular composition,

TABLE I. Summary of the most commonly used commercially available methods for single nanoparticle analytics.

Single nanoparticle methods	Physical parameters determined	Size in diameter	Concentration (volume)[d]
Electron microscopy (EM) *cryo-frozen solution based*	Size, shape	~1–10 nm	10^{12}–10^{14} particles/ml (~2 μl)
Nanoparticle tracking analysis (NTA) *solution based*	Hydrodynamic diameter, charge, optical fingerprint,[a] concentration[b]	~30–60 nm	10^{7}–10^{9} particles/ml (~750 μl)
Resistive pulse sensing (RPS) *solution based*	Size, charge, electrical fingerprint,[c] concentration	~30–60 nm	10^{7}–10^{9} particles/ml (~1 ml)
Flow cytometry *solution based*	Size, optical fingerprints, concentration	~50–100 nm	10^{7}–10^{9} particles/ml (~1 ml)
Optical microscopy *surface based*	Optical fingerprint	~5–40 nm	10^{11}–10^{13} particles/ml (10–100 μl)

[a]Optical fingerprint refers to the possibility to identify one or several components of the nanoparticles using, e.g., fluorescent markers.
[b]Requires that all nanoparticles with a known volume generate a detectable signal.
[c]See Sec. III.
[d]Since the concentration can usually be controlled by either diluting or concentrating the sample, we here provide both the typical concentration and sample volume used in a single analysis.

and content required to be taken up by and subsequently reprogram neighboring cells. Similarly, for many types of viruses, it is well established that only a small fraction are infectious and thus capable of spreading disease.[25]

Providing analytical information about single biological nanoparticles has traditionally been restricted to electron microscopy (EM), which offers a resolution that approaches the detection limit in terms of nanoparticle size of many ensemble-averaging methods (see Table I). Soft biological nanoparticles of heterogeneous nature can be analyzed using transmission EM (TEM), providing information about both dimension and structure of individual nanoparticles; however, the transfer of the nanoparticles to a solid substrate and the staining required prior to analysis under vacuum conditions tend to cause structural alterations that complicate proper analysis.[26] Cryo-freezing prior to TEM (cryo-TEM) analysis is thus strongly preferred in order to obtain representative structural information of soft nanoparticles.[27] An additional and particularly valuable asset of TEM is the possibility to combine structural and dimensional heterogeneities with gold nanoparticle labeling of specific biomolecular entities, e.g., immune-gold labeling. In a recent study by Brisson *et al.*, cryo-TEM was successfully combined with both antibody and annexin-V labeled gold nanoparticles,[28] making it possible to, for example, identify subpopulations of certain structures and sizes exposing either a specific protein receptor or phosphatidylserine lipids or both. Although high-resolution cryo-TEM is quite informative, it remains a challenge to obtain instantaneous information with high throughput in a convenient manner. In addition, the analysis is costly and must often be carried out in shared microscopy centers and suffers from limitations with respect to low statistics and complications arising as a consequence of, often cumbersome, preparation protocols.

In contrast to EM, atomic force microscopy (AFM) can be carried out at room temperature and in the wet state, but the soft nanoparticles must be immobilized to an interface prior to analysis. Although one must keep in mind that both surface immobilization and the close proximity between the sample and the tip used for imaging may impact structural integrity, liquid-phase AFM has been shown to provide information about topography as well as mechanical and chemical properties of soft nanoparticles.[29–31] Further, by modifying the AFM tip such that it interacts specifically with biomarkers on the surface of nanoparticles, it has been verified that force spectroscopy curves could discriminate different subpopulations of extracellular vesicles.[31] By modifying the substrates with binders, such as antibodies, directed against markers on the surface of biological nanoparticles, one should, in analogy with ensemble-averaging surface-sensitive methods such as SPR (Ref. 32) or nanoplasmonics,[23,33] be able to identify specific subpopulations of nanoparticle suspension via selective binding to the surface. However, quantification of the concentration of surface markers on biological nanoparticles is not straightforward with any of these approaches. Furthermore, despite offering valuable biophysical and structural insights, AFM suffers, much like EM, from cumbersome preparation protocols and extended analysis times. As a consequence, statistics are often based on a low sample size, which has a direct influence on the ability to address statistical distributions in heterogeneous samples. Although the number of particles that should be analyzed to gain sufficient statistics with either EM, AFM, or alternative single nanoparticle tools, varies widely depending on the information one strives for, it generally requires somewhere in the range of a couple of hundred to a few thousand particles. As this is often time consuming to obtain using AFM and EM, and as this is an aspect of significant importance in single nanoparticle analytics, the need for alternative bioanalytical tools offering immediate information and good statistics persists.

Thanks to ease of use and the capacity to provide a wide range of detailed information, optical microscopy has become the most important imaging method in all of life science. With appropriate labeling strategies, fluorescence

microscopy contributes invaluable information regarding the biological impact of nanoparticles; however, whether of biological origin or not, nanoparticles are not naturally labeled with fluorophores, and incorporation of labels inevitably adds an additional preparative step and might influence their properties and function. Means to perform label-free optical single nanoparticle analysis is thus of high importance but, due to their very weak optical contrasts, this is a very demanding task. In order to utilize light scattering in nanoparticle analysis, it is a prerequisite to efficiently suppress background scattering, which can be most easily achieved by letting the nanoparticles diffuse freely in a perfectly dust-free aqueous solution. Modern optics, then, makes it possible to image and track the motion of individual nanoparticles while diffusing through a volume illuminated by a collimated light beam. Under the assumption that all nanoparticles in a known illumination volume are detected, it is possible to quantify both concentration[34] and size;[35] the latter by determining the diffusion constant ($\propto$1/radius) from SPT analysis. For example, despite not being able to detect optically faint and small (radius $<\sim$20 nm) particles, nanoparticle tracking analysis (NTA) has been shown to provide accurate information about the hydrodynamic size as well as size distribution of heterogeneous samples. Combined with ease of use and low sample consumption, see Table I, this explains the growing popularity of NTA over the past couple of years.

NTA has been shown to correlate very well with alternative methods designed for size determination of individual nanoparticles, such as tunable resistive pulse sensing (TRPS),[36] which in analogy to the nanopore sensing concept developed by Mayer *et al.* (described further in Sec. III) takes advantage of the temporal evolution of a resistive pulse in an ionic current generated upon passage of individual suspended nanoparticles across a nanoscale aperture in a thin membrane. In analogy with immunogold labeling combined with EM analysis, it is possible to implement NTA not only in scattering but also in fluorescence mode such that molecular labeling approaches (e.g., immuno-labeling) can enable the identification and analysis of biomolecular nanoparticles with specific molecular content.[37] In this way, molecular fingerprinting can be correlated with nanoparticle size; for instance, the size of CD63 positive EVs derived from human mast cells was shown to have a size distribution of around 80 nm, which was significantly smaller than the mean size of $\sim$200 nm of the corresponding EVs determined in scattering mode.[22] In future designs by implementing multispectral fingerprinting, which is today routine in flow cytometry analysis of cells, the biological significance of this mode of operation will further increase as it will harness the capacity for combinatorial fingerprinting. The current version of NTA has one significant drawback, however, which is that the nanoparticles under analysis diffuse in and out of a collimated illumination beam, thus making the intensity of the emitted light subject to significant fluctuations. Therefore, quantitative determination of the scattering intensity and/or the fluorescence emission becomes very uncertain, thereby complicating the possibility to quantify, on the single nanoparticle level, the molecular content and its correlation with particle size.[38–40]

To address the latter challenge, Höök *et al.* have developed, as presented at BI 2016, a method for making it possible to simultaneously quantify both the size and the molecular content of individual nanoparticles.[4] The concept utilizes the surface localization and reduction in diffusivity obtained by tethering nanoparticles to a fluid supported lipid bilayer (SLB), which fulfills the purpose of confining the mobile nanoparticles to the focal plane for optimal microscopic visualization. In this method, referred to as *2-dimensional nanometry*, both the fluorescence emission and scattering intensities (possible by suppressing interfacial scattering) can be determined with high accuracy,[41] which is illustrated in Fig. 2 for fluorescently labeled lipid vesicles (see legend to Fig. 2 for details).

By also inducing a directed motion of the tethered nanoparticles using a shear flow, the force acting on the nanoparticles can be quantified from their directed drift velocity since the drag (inversely proportional to the diffusivity) represents the proportionality constant between force and velocity. Hence, owing to the possibility to decouple the stochastic Brownian motion [y-axis in Fig. 3(b): diffusivity] of individual nanoparticles from their deterministic shear-flow-induced motion [x-axis in Fig. 3(b): velocity], microscopic visualization and SPT analysis of nanoparticles tethered to a supported lipid bilayer was shown to enable determinations not only of optical fluorescent fingerprints but also of the force acting on them. Furthermore, since the dependence of the measured force on the size of the nanoparticles can be expressed analytically,[40] the individual nanoparticle size could be determined with high accuracy in a diameter interval of 30–300 nm. Further, in this interval the fluorescence intensity of individual lipid vesicles was indeed shown to display significantly better agreement with particle size than that obtained for the same particles using NTA [Fig. 3(c)], potentially opening up a new avenue in single nanoparticle analytics.

Previously, correlations between nanoparticle size and fluorescence intensity have been reported for artificial lipid vesicles, in which case one type of fluorescently labeled lipid was used to report on particle size (fluorescence emission $\propto r^2$) and another to report on molecular content. Even for synthetic lipid vesicles, surprisingly large heterogeneities in molecular (lipid) distribution were reported using this approach.[42] In fact, the 2D flow nanometry data shown in Fig. 3 suggest that there is a significantly lower heterogeneity than previously reported and thus indicates that independent determinations of nanoparticle size and molecular composition offer improved analytical precision; a feature that will be even more relevant in the case of biologically derived nanoparticles for which it is hard to identify the marker needed to report on their size. While size and molecular content of individual nanoparticles in heterogeneous samples are obviously very relevant parameters, even more would be gained if additional properties, such as shape and charge, could be simultaneously determined. In fact, as summarized in Sec. III, recent advances in nanopore sensing have been

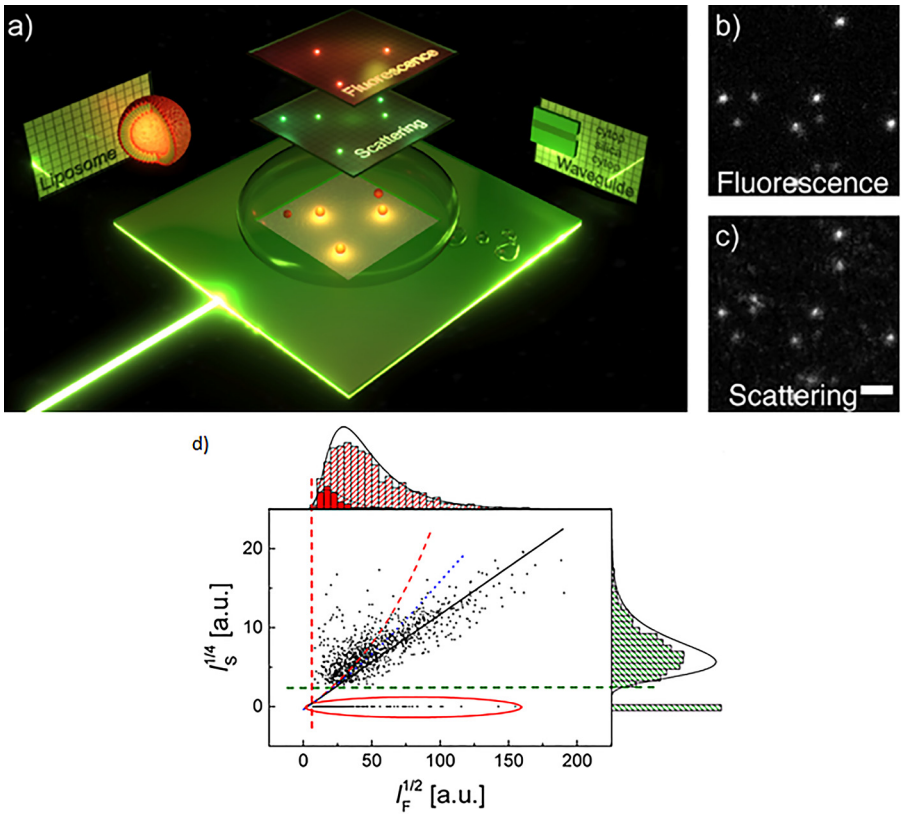

FIG. 2. Schematic representation of the planar waveguide chip and detection of fluorescently labeled vesicles in fluorescence and scattering modes. (a) A single mode optical fiber was aligned to the facet of the planar waveguide and light (green) was coupled into the core layer of the chip. An opening (sample well) was formed in the upper cladding layer of the waveguide, into which a solution (drop) containing the specimen of interest was placed. The evanescent part of the in-coupled light interacts with objects present within the penetration depth of the evanescent light resulting in light scattering and, in the case of fluorescent objects, fluorescence generation. The emitted light was collected using a standard microscope objective in an upright or inverted configuration, or a combination of both, to reveal a scattered signal in the scattering image plane (green square) and/or a fluorescence signal in the fluorescence image plane (red/orange square). (b) Fluorescence and (c) scattering signals from the same $12 \times 12\,\mu m^2$ area of the chip under identical illumination conditions, showing nominally 150 nm fluorescently labeled vesicles bound to the surface imaged with high- and low-pass filters, respectively, with cutoff wavelength of around 550 nm, i.e., in between the excitation (532 nm) and emission (582 nm) wavelengths. The scale bar in (c) corresponds to $2\,\mu m$. (d) Scattering and fluorescence intensities from single vesicles. Scaling with the powers 1/4 and 1/2 for 2000 single fluorescently labeled vesicles with an average diameter of 150 nm. The plot is expected to be linear provided $I_s \propto r^4$ and $I_f \propto r^2$. The distributions of the scattering (green) and fluorescence (red) intensities have been projected onto the y and x-axis, respectively, and correspond to the size distribution of the vesicles. The red vertical and green horizontal dashed lines indicate the limit of detection. The black straight line, red dashed curve, and blue dotted curve show the theoretically predicted behavior. Adapted with permission from B. Agnarsson, *et al.*, *ACS Nano* **9**, 11849 (2015). Copyright 2015, American Chemical Society.

recently shown to provide exactly this not only for biological nanoparticles but also for individual protein molecules.

III. SINGLE PROTEIN ANALYTICS: NANOPORE-BASED RESISTIVE PULSE SENSING

To improve the limit of detection in medical diagnostics and in routine protein assays, the ultimate vision would be a quantitative and descriptive analysis of individual proteins in complex mixtures *without* the need for antibodies or other affinity reagents as selective capturing agents. Characterization, identification, and quantification of individual proteins in complex solutions without the use of specific labeling strategies is, however, extremely demanding. State-of-the-art mass spectrometry, which is arguably the most powerful technique for label-free protein analysis,[43–45] requires femtomole amounts (10^{-15} mol) of proteins for accurate detection and can therefore not provide information about the structure or shape of individual proteins. The only techniques that can reveal detailed shape information from single proteins and protein complexes are cryo-transmission electron microscopy (cryo-TEM or cryo-EM) and to a lesser extent AFM has been explored for imaging protein structure in a physiological medium. Among these, cryo-EM has proven capable of unraveling protein structures at atomic resolution—an astonishing feat that has been recognized with the Nobel Prize in Chemistry in 2017. However, cryo-EM typically determines protein shape from class averages obtained from analysis of several thousand randomly oriented particles from a pure sample; the method therefore analyzes individual proteins but the final result originates from averages of many proteins.[46,47] AFM, on the other hand, can provide image and shape information from single proteins and oligonucleotides; however, the biomolecules of interest have to be immobilized on an atomically flat surface and, due to the finite sharpness of AFM tips, the resolution of the

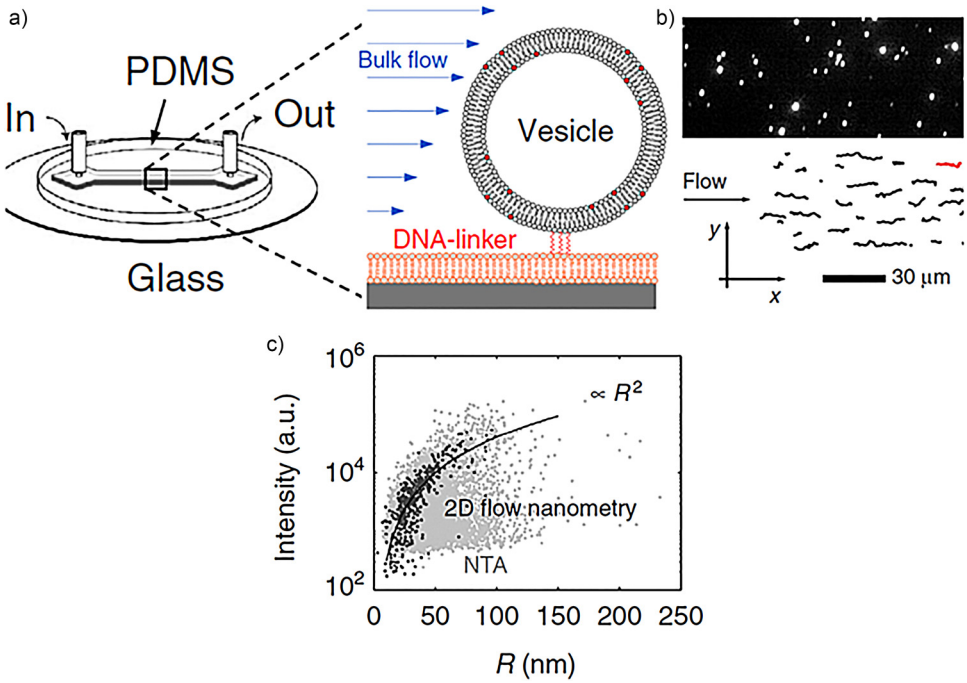

FIG. 3. (a) Small unilamellar vesicles (SUVs) linked to an SLB at the floor of a microfluidic channel by cholesterol equipped DNA tethers subjected to a shear flow in a microfluidic channel. (b) Single particle tracking analysis provides information about diffusivity (number of linkers) and shear-induced drift velocity. (c) Comparison of vesicle intensity extraction done by 2D flow nanometry and NTA using intensity versus size parameter plots obtained by NTA (gray dots) and 2D flow nanometry (black dots) for SUVs. Thanks to much lower intensity fluctuations observed in 2D flow nanometry with respect to NTA, the expected scaling law ($\propto r^2$) is well visible in the parameter plots [solid line in (c)], while it is hard to resolve for NTA data. Adapted with permission from S. Block, B. J. Fast, A. Lundgren, V. P. Zhdanov, and F. Höök, *Nat. Commun.* **7**, 12956 (2016). Copyright 2016, Nature. See also Creative Commons Attribution 4.0 International License, https://creativecommons.org/licenses/by/4.0/.

technique is limited to revealing the location of local domains rather than atomic structure.[48,49] Other techniques for determining the shape of proteins such as x-ray crystallography,[50] analytical ultracentrifugation,[51] NMR spectroscopy,[52] or short angle x-ray scattering[53] either require a protein crystal or are ensemble techniques, which are limited to determining parameter averages. Further, with the exception of analytical ultracentrifugation, all these techniques require samples of extremely pure protein and their performance decays with increasing size of the protein, protein complex, or nanoparticle.

Based on these limitations, there exists a need for broadly accessible techniques to characterize single particles ranging from individual protein molecules to hierarchical nanoparticle assemblies with regard to their size, shape, and state of assembly. An important step towards the possibility to detect and discriminate different types of proteins was demonstrated by Mayer *et al.*, as presented at the BI 2016 meeting, thereby allowing not only the charge, size, and rotational diffusion coefficient of *individual* proteins to be simultaneously determined but also an approximation of their molecular shape and dipole moment. Importantly, these characterizations occurred in aqueous solution under nondenaturing conditions.[5,54] As Fig. 4 illustrates, the approach is based on probing temporal changes in the ionic conductance across nanoscale apertures during passage of individual molecules, a concept that was so far primarily used for DNA sequencing applications, as recently reviewed,[55–57] as well as for nanoparticle analysis using TRPS.[36] In contrast to sequencing DNA, which thus far employs biological nanopores through integral transmembrane proteins with diameters smaller than 2 nm, characterization of folded, globular proteins requires larger pore diameters in the range of 5–50 nm typically fabricated in silicon nitride membranes.[5,58] One challenge with inorganic substrate materials is, however, adsorption of proteins to the nanopore walls. To minimize these interactions, inspiration was gained from nanopores in the exoskeleton of insects [Fig. 4(a)] by coating the walls of the nanopores with a fluid SLB[54] and is discussed further in Sec. VI. In analogy with the 2D flow nanometry method presented in Sec. II, the two-dimensional fluidity of the SLB provides, on the one hand, a nonstick, nonfouling surface[59] and, on the other hand, the opportunity to include lipid anchors to which suspended proteins, typically in a nanomolar concentration range, can be tethered via flexible linkers [Fig. 4(b)].[54] For instance, lipids displaying biotin groups on their headgroups make it possible to capture, concentrate, and analyze biotin-binding proteins on the surface.[54] This concept was also extended to a sandwich assay in which lipid-anchored streptavidin was used to bind a commercially available, biotinylated anti-catalase antibody in order to specifically capture and quantify catalase, thus illustrating the capacity to be expanded to a multitude of proteins.

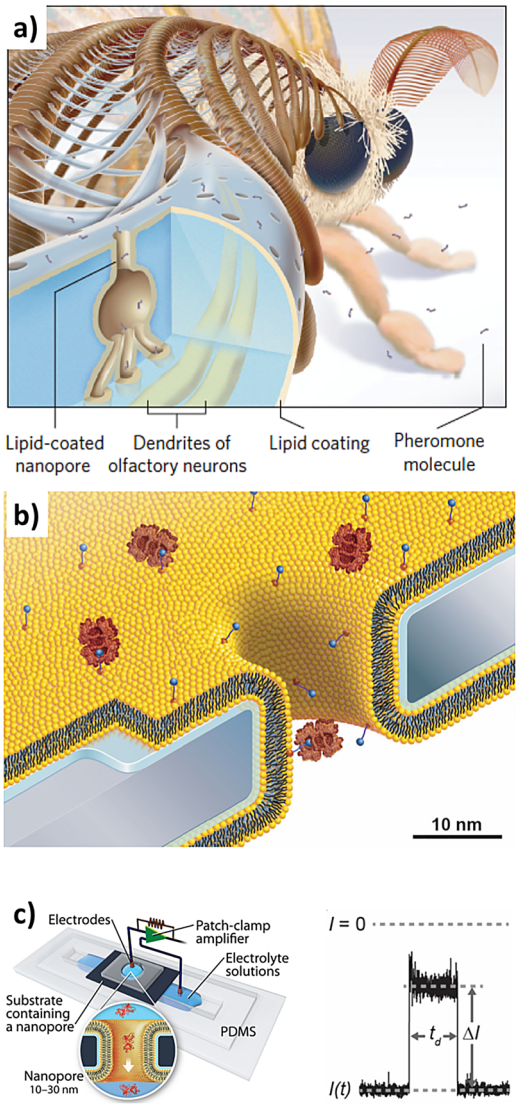

FIG. 4. Bioinspired nanopore with fluid lipid bilayer coating to facilitate the movement of single molecules or nanoparticles through the pore combined with characterization of the particle based on resistive pulse recording. (a) Nanopores through the exoskeleton of the silk moth *Bombyx mori* are coated with a fluid lipid or wax coating to facilitate the capture and diffusion of pheromone molecules toward receptors on the dendrites in the antennae of the moth. (b) Cartoon of a cross-section through a solid state nanopore fabricated in a silicon nitride window and coated with a supported phospholipid bilayer. The layer in light blue indicates the water layer between the solid substrate and the lipid bilayer membrane. Lipid anchors (dark blue spheres) capture and concentrate specific proteins on the surface. (c) Experimental setup of a resistive pulse recording experiment and typical resistive pulse signal from translocation of a spherical particle through the nanopore. The presence of a particle in an electrolyte-filled pore displaces conducting electrolyte and transiently reduces the absolute magnitude of the baseline current through the pore. The amplitude of the current blockade ΔI is proportional to the volume of the particle, the duration of the blockade is inversely proportional to the charge of the particle and the frequency of blockades is proportional to the concentration of the particle. Adapted with permission from E. C. Yusko *et al.*, *Nat. Nanotechnol.* **6**, 253 (2011). Copyright 2011, Springer Nature. Also adapted from E. C. Yusko *et al.*, *Nat. Nanotechnol.* **12**, 360 (2017). Copyright 2017, Springer Nature.

Since nanopore experiments are based on the exclusion of conducting electrolyte by any particle in the pore [Fig. 4(c)], this technique is not limited to the analysis of monomeric proteins; it extends to multimeric proteins,[5] protein complexes,[5,59,60] viruses,[61] and nanoparticles.[62] To perform a meaningful analysis of current modulations during a resistive pulse from the translocation of a single protein or particle, it is, however, critical that these current modulations are resolved in time and that the supramolecular complex resides in the pore sufficiently long to adopt various orientations before it exits.[5] This requirement is met by taking advantage of the viscosity of the fluid lipid bilayer coating on the nanopore walls. Specifically, the lipid anchor slows down particle translocation due to increased drag by the approximately 100-fold higher viscosity of lipid bilayers compared to the viscosity of the aqueous electrolyte.[5,54] Using this lipid anchoring approach, the Mayer group recently demonstrated that the passage time through the nanopore can become sufficiently prolonged to time-resolve modulations of ionic currents that are related to protein shape and orientation. This fine structure of resistive pulses can be correlated with multiple protein properties, including volume, shape, rotational diffusion coefficient, charge, and dipole moment.[5]

Figure 4(c) shows an experimental setup for nanopore-based resistive pulse recordings from translocations of single proteins through the pore and serves to illustrate the concept of characterizing individual nanoparticles. This setup contains two reservoirs that are filled with aqueous electrolyte solution and separated by a thin, insulating membrane. Low-impedance Ag/AgCl electrodes in both reservoirs connect them to a high gain, low-noise amplifier that applies a constant potential difference while measuring the ionic current through the nanopore. The crucial concept of this single molecule technique is that almost the entire voltage drop in the conducting pathway occurs inside the nanopore, rendering this zone supremely sensitive to changes in its ionic conductivity. Any particle entering this volume displaces highly conducting electrolyte and increases the pore's resistance during its translocation. If the pore volume is on a similar size scale as the volume of individual proteins or nanoparticles of interest, then the resulting reduction in current amplitude from the baseline current, ΔI, is readily detectable.

For spherical particles, the current blockade ΔI is directly proportional to the electrolyte volume excluded by the particle and as long as a particle's volume is sufficiently large to displace at least 1% of the electrolyte volume in the pore, it is detectable. This means that the detection limit with regard to the smallest detectable proteins or particles can be adjusted by appropriate choice of the nanopore volume. For instance, pores in biological ion channel or porin proteins with diameters close to 1 nm have been used to detect individual small organic molecules without the requirement of labeling these molecules.[63] Figure 5 demonstrates this concept on the level of single virus particles, gold nanoparticles, and protein amyloids. For virus particles, nanopore-based resistive pulse sensing can be used to characterize individual viruses with regard to size, time-dependent increase in size upon binding of antibodies, binding constant of these antibodies on the native virus particle in solution, and number of epitopes on the virus surface [Figs. 5(a) and 5(b)].[61] Based on these capabilities, and those above, it is

possible to imagine the richness of information about individual nanoparticle size, structure, and content that could potentially emerge by combining the approaches of Mayer and Höök on the same chip; the slower motion caused by tethering nanoparticles to a supported membrane extends the duration of the passage time through a pore, thereby increasing signal-to-noise, which could enable structural information to be directly correlated with complementary information about hydrodynamic size and nanoparticle content obtained using 2D flow nanometry (see Sec. II).

Self-assembled nanoparticles are often heterogeneous in size, shape, and content, rendering ensemble-based analysis techniques such as DLS inefficient for their characterization, as discussed in Sec. II. A dramatic example of heterogeneous particles are protein amyloids, which are relevant in the context of several neurodegenerative diseases such as Alzheimer's and Parkinson's disease [Fig. 5(d)]. In order to interrogate heterogeneous samples, the nanopore-based characterization approach takes advantage of two characteristics: first, the ultrasmall sensing zone, which can only accommodate one or a few particles at a time and, second, the short translocation time through this small volume, which is on the order of microseconds. This short translocation time combined with an average translocation frequency of approximately 10 Hz means that the probability of two particles entering the sensing volume at the same time is very low. In addition, the translocation frequency can be reduced by diluting the analyte to further reduce the probability for double occupancy in the pore. On the other hand, if two or more particles or molecules interact with each other to form a complex, then these complexes will enter the pore as one particle and we showed previously that such complexes remain intact under typical experimental conditions.[5] In the case of weak interactions, this may not be the case and the disassembly of such complexes may occur during the actual translocation event. By probing multiple events of this nature, it may thus be possible to investigate the dynamics of weak complexes by monitoring, during each translocation event, for instance, the shape of particles, which is likely to change dramatically during disassembly. These examples illustrate that the single particle analysis capability of nanopore-based experiments offers new opportunities for in-depth characterization of the heterogeneity of engineered nanoparticles and shows promise for characterizing

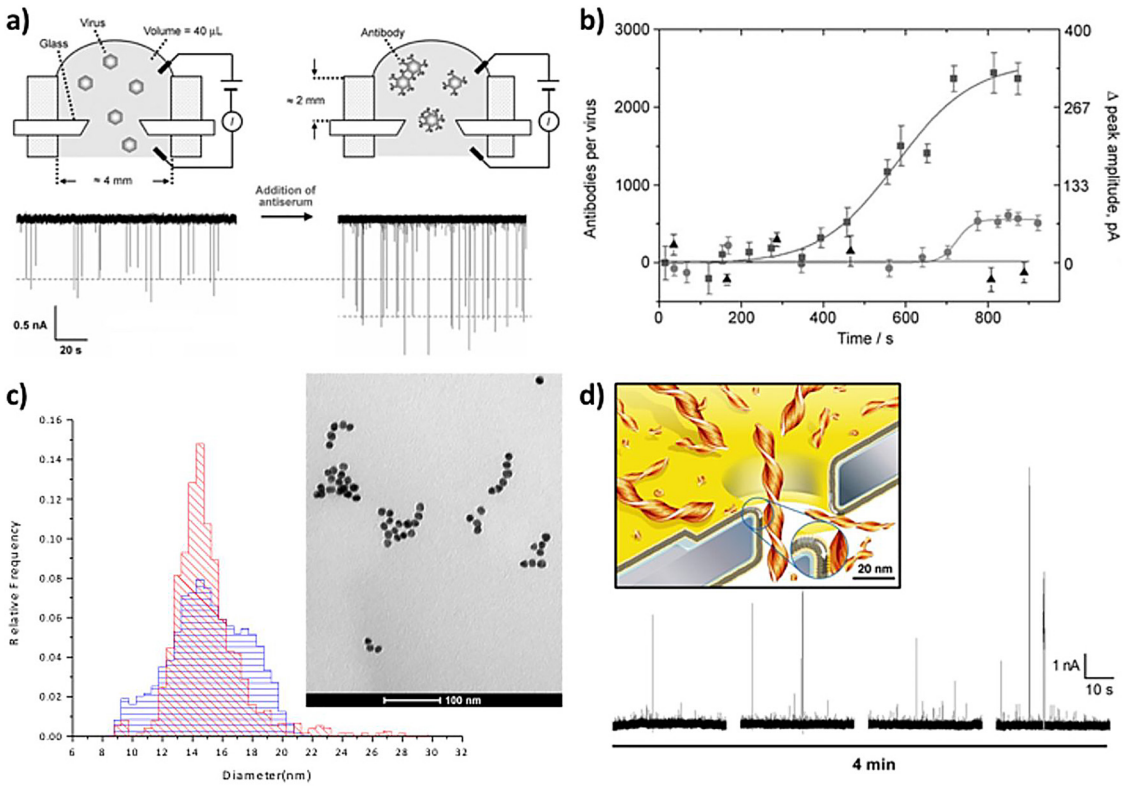

FIG. 5. Nanopore-based analysis of virus particles, gold nanoparticles and protein amyloid aggregates. (a) Resistive pulses before and after addition of antiserum to a solution of viruses make it possible to quantify their size with and without antibody coating. (b) Monitoring the time-dependent increase in antibody size upon antibody binding reveals the affinity of the antibody on intact virus particles in solution and determines the maximum number of antibodies that can bind to a virus particle. (c) Comparison of the size distribution determined in solution with nanopore-based analysis (blue) with the size distribution of the same sample determined by electron microscopy (red and inset). (d) Nanopore-based resistive pulse analysis of amyloid-beta (Aβ) aggregates showing the increasing amplitude of resistive pulses as the aggregates grow during the analysis. The inset illustrates that amyloids can only be characterized in a solid state nanopore if the walls are coated with a nonstick fluid lipid bilayer coating (otherwise the pore would clog within seconds). Adapted with permission from J. D. Uram, K. Ke, A. J. Hunt, and M. Mayer, *Small* **2**, 967 (2006). Copyright 2006, John Wiley and Sons. Also adapted with permission from E. C. Yusko *et al.*, *Nat. Nanotechnol.* **6**, 253 (2011). Copyright 2011, Springer Nature.

the complex, hierarchical assemblies that are often required for targeted drug delivery.

In addition to determining the volume of viruses and proteins, nanopore-based resistive pulse sensing can also be used to determine the approximate shape of individual proteins.[5] To do so, the approach takes advantage of the Brownian rotational dynamics of particles inside the nanopore. As shown in Fig. 6(a), a lentil-shaped object (oblate) distorts the electric field lines more significantly if its flat side is oriented perpendicular to the pore axis, and hence the electric field, than when it is oriented parallel to the pore axis. Similar arguments can be made for a rugby ball shaped object (prolate), which also causes the largest current blockade in its cross-wise orientation. The magnitude of the particle-induced current blockade is therefore a function of protein volume, protein shape, and time-variant protein orientation during its journey through the pore.[5] By time-dependent analysis of the ΔI signal as a particle moves through the pore, the approach makes it possible to determine the change in orientation over time, revealing the rotational diffusion coefficient in addition to the particle's volume and ellipsoid approximation of its shape.

Moreover, since the orientation of a particle in the extremely strong electric field (~MV m^{-1}) inside a nanopore is biased by the permanent dipole moment of the protein or particle, analysis of this bias in orientation during translocation reveals the magnitude of the particle's dipole.[5] Since established approaches to measure dipole moments of proteins, such as dielectric impedance spectroscopy are limited to ensemble measurements,[65] precise determinations of the dipole moments of *individual* proteins and nanoparticles could add valuable information regarding heterogeneity originating from, for example, various post-translational modifications such as phosphorylation and glycosylation. Although dipole interactions are weak, the dipole moment may indeed be an undervalued descriptor for particles and proteins. For example, the absolute protein dipole moments range from zero to a few thousand Debye among different proteins.[5] Hence, dipole moment, if measured with sufficient precision, could offer a more powerful protein discriminator than net charge, which typically remains within 10–20 unitary charges.[5] Despite its weak and short-range nature, the dipole moment is also an important parameter to consider in molecular assemblies, since dipole alignment and interactions are known to influence the orientation of molecules and the stability of assemblies as well as the rheological properties of solutions of these particles.[5]

While nanopore-based resistive pulse recordings offer a unique means to determine the dipole moment of nanoparticles, these experiments also enable determination of the net charge. Instead of measuring the drift velocity of proteins and nanoparticles when subjected to an electric field, this analysis considers the time it takes for a particle to translocate the length of the nanopore, which is called dwell time, t_d, and related to the particle's electrophoretic mobility and hence its net charge.[5] Nanopore-recording can therefore characterize single proteins and nanoparticles in aqueous solution with regard to five parameters that are relevant for

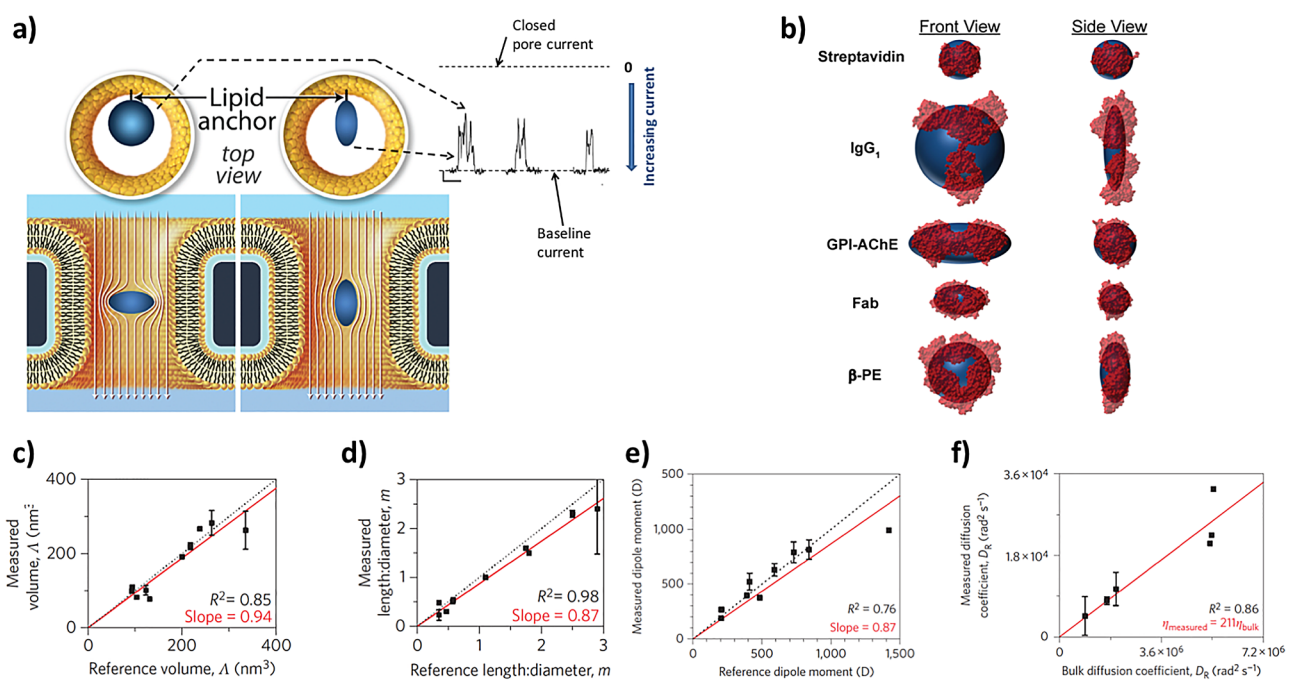

FIG. 6. Analysis of current modulations while particles translocate and rotate through a nanopore makes it possible to determine the volume, shape, dipole moment and rotational diffusion coefficient of the particle. (a) Nonspherical particles modulate the resistance of a nanopore as a function of the shape and orientation of the particle in the electric field of the nanopore. (b) Comparison of the crystal structure of five proteins (red) with an ellipsoid approximation of their shape as determined by single molecule nanopore recordings (blue). [(c)–(f)] Comparison of the particle volume, shape, dipole moment and rotational diffusion coefficient measured in a nanopore with reference values, illustrating good agreement. Adapted with permission from E. C. Yusko *et al.*, *Nat. Nanotechnol.* **12**, 360 (2017). Copyright 2017, Springer Nature.

supramolecular assemblies: volume, shape, rotational diffusion coefficient, dipole moment, and charge. The fidelity of this multiparameter characterization is illustrated in Figs. 6(c)–6(f), which shows the correlation between the measured particle volume, shape, dipole moment, and rotational diffusion coefficient with the corresponding reference values.

With regard to the characterization of nanoparticles and supramolecular assemblies, this section and the 2D flow nanometry concept presented in Sec. II illustrate that methods are emerging with certain advantages for determination of multiple parameters providing a fingerprint analysis of individual nanoparticles and rare biological molecules in complex and aqueous solutions (Fig. 6). In this context, it is important to realize that the parameters, which make up the fingerprint, are obtained simultaneously from each resistive pulse when a single protein or a single protein complex translocates through the nanopore.[5] For instance, the time-averaged magnitude of the resistive current blockade is proportional to the volume of the particle, the duration of the resistive pulse to the net charge of the particle, the current "noise" during the resistive pulse is related to the particle's shape and orientation in the electric field, the frequency of these current modulations to its rotational diffusion coefficient, and the bias in these modulations towards one orientation to its dipole moment.[5] Changes in particle properties such as volume, shape, or charge can therefore be determined unambiguously because the various measured properties relate differently to these particle properties. These novel, single particle characterization approaches therefore yield the stoichiometry of assemblies, the number of accessible surface groups or epitopes, the kinetics of assemblies and disassemblies, the stability of complexes, and a low-resolution ellipsoid approximation of their shape as required to design and optimize, for example, supramolecular assemblies of next generation drug-delivery vehicles.[60,61] While living cells excel at assembling macromolecular subunits to functional delivery systems in the form of exosomes or viruses, engineering artificial nanoparticles with these capabilities is challenging because of multiple possible failure points on the assembly trajectory. Thus, progress in designing and fabricating such hierarchically assembled nanoparticles reliably in bulk, as required for therapeutic use, requires accurate and fast techniques for quality control. The need for such progress in nanoparticle analytics is further illustrated in Sec. IV, which places emphasis on recent advancement in the design of stimuli-responsive synthetic polymer-based nanoparticles designed for either oligonucleotide/protein delivery or to function as artificial organelles.

IV. BIOMIMETIC DRUG-DELIVERY ASSEMBLIES AND ARTIFICIAL ORGANELLES

Conventional low-molecular-weight drugs typically execute their tasks by interacting with and thereby influencing the function of specific proteins or by detoxifying harmful agents inside cells, while the next generation drugs are expected to be increasingly based on high-molecular-weight biologicals designed to reprogram the cells from within,[14,66] as well as being based upon functional supramolecular biosynthetic assemblies intended to serve as cellular implants such as artificial organelles.[6,67] In contrast to conventional drugs, but in analogy with the genetic cargo carried by exosomes and viruses, many of these biological drugs (peptides, proteins, oligonucleotides, etc.) do not spontaneously translocate across the cell membrane. Therefore, an elegant strategy to design next generation nanoscopic delivery vehicles is a bottom-up, bioinspired strategy that mimics naturally evolved systems. Significant scientific effort, over the past several decades, has resulted in a large variety of such delivery vehicles including virus-mimicking protein capsids, lipid- and polymer-based micelles, lipid- and polymer-based vesicles, and polymer nanoparticles; each providing specific architecture and properties to host biological molecules.[6,68–70] The desired biological molecules (oligonucleotides, polypeptides, enzymes, and proteins) can be entrapped, membrane inserted, and/or attached to the surface depending on their intrinsic physicochemical properties, such as shape, hydrophilicity, dipole moment, charge, flexibility, etc. Preferably, the carriers are also equipped with a cell-membrane homing moiety, such as an antibody, a peptide binder, or specific receptors, designed to target a specific cell type.[6,14,16,71]

Despite significant efforts, the most efficient delivery systems today, based on biomimetic amphiphilic 3D assemblies, demonstrate a very low functional delivery efficiency. There are many reasons as to why high-molecular-weight cargo delivery using artificial systems does not lead to the intended functional response in the target cells, all of which relate to a limited understanding of certain key processes. For example, although it is an active area of research, it remains a challenge to control the physicochemical match required between molecular carrier components and their drug cargo in order to avoid the uncontrolled release into nondesired bioregions. Another issue is the lack of understanding required to control ligand–receptor dynamics and geometrical positioning at the interface between nanocarriers and the cellular membrane in order to induce, for example, uptake via endocytosis. Another crucial process that must be successfully controlled in order to generate a functional biological response is the efficient release of the biological drug cargo from the endosomes prior to their degradation. Means to improve this so-called endosomal-escape step are often considered one of the key bottle necks in improving the performance of drug-delivery nanocarriers, especially for lipid based systems.[3,72]

A number of strategies are being explored to develop more efficient vehicles; one promising approach is to render polymer-based nanocarriers, in particular, to be stimuli responsive such that they will release their cargo only upon changes in the bioenvironment.[73,74] Palivan *et al.* have developed, as presented at BI 2016, stimuli-responsive synthetic nanoparticles for protein delivery based on asymmetric poly(ethylene glycol)-*b*-poly(methylcaprolactone)-*b*-poly(2-

(*N*,*N*-diethylamino)ethyl methacrylate) (PEG-*b*-PMCL-*b*-PDMAEMA) copolymers that allow both a controlled entrapment of the proteins and a *p*H-sensitive release under variable cellular conditions.[75] Such stimuli-responsive vehicles are a significant improvement toward optimized functional drug delivery, demonstrating improved temporal and spatial precision in comparison to conventional delivery carriers. However, an important issue to be overcome is the controlled release of payload at the desired biosite when passing through other sites presenting the same biostimulus. Advances are expected through efforts to develop appropriate combinations of stimuli-responsive delivery vehicles and targeting molecular groups for the improved spatial precision in payload delivery. Furthermore, significant improvement in the efficacy of stimuli-responsive delivery carriers is expected, benefiting from further systematic *in vitro* and *in vivo* assays evaluating the internalization mechanisms, biodistribution and release, together with advances in analytical techniques such as the ones presented herein.

Recently, a complementary strategy to provide a desired functionality to the cell interior has been introduced by the design of catalytic compartments, named nanoreactors[6,76,77] as well as the first examples of artificial organelles mimicking natural organelles *in vitro* and very recently *in vivo* (Fig. 7).[78,79] Nanoreactors are based on encapsulation/entrapment of active compounds (enzymes, proteins, mimics of proteins) inside supramolecular assemblies such that the active compounds fulfill their activity *in situ*, without being released.[6,76,77] Particularly appealing for generation of nanoreactors are amphiphilic block copolymers because, if appropriately selected in terms of their chemical nature and properties, they overcome the instability of liposomes, while being able to mimic lipid membranes in terms of their low immunogenicity.[80] In addition, it is possible to modulate the properties of such synthetic compartments (size, stimuli-responsiveness, permeability) by the intrinsic nature of the selected copolymers and the hydrophobic-to-hydrophilic ratio.[81] Various nanoreactors have been reported as model systems, demonstrating a number of reactions that can be carried out in confined spaces by encapsulated single enzyme types,[76,82] as well as when multiple enzyme types were encapsulated/inserted in specific regions of nanocompartments [Figs. 7(a)–7(c)].[77,78,83] For example, both natural enzymes, such as laccase, and artificial ones were shown to catalyze substrate conversion in the interior of polymersomes.[82,84] Protein-containing compartments have been developed for various medical applications including the production of antibiotics[85] or metabolites, as, for example, glucose-6-phosphate[86] as a source of oxygen species for photodynamic therapy and for oxygen transport and peroxynitrite detoxification.[87,88] As the chemical reaction is to be performed *in situ* (i.e., inside the compartment cavity), a challenging factor is the permeability of the compartment membrane to allow a molecular flow through (e.g., for both substrates to enter the inner cavity and for products to be released from within). In the quest for selective permeability, various approaches for obtaining permeable membranes have been explored including (1) using a block copolymer, which forms a porous membrane,[89] (2) blending block copolymers with lipids followed by extraction of the latter,[90] (3) chemical modification of the membrane to create pores or allow molecular diffusion,[82] and (4) reconstitution of wild-type channel proteins and genetically modified proteins to provide a stimuli-responsive "open"/"close" mechanism to the pores [Figs. 7(d) and 7(e)].[79,91,92]

A step further in the development of functional assemblies has been achieved by encapsulation of different enzymes working in tandem inside synthetic compartments in a way that mimics native organelles. The *in situ* cascade reactions of artificial organelles serve to produce a desired compound or to restore healthy cellular conditions.[78,93] Compared to nanoreactors, artificial organelles have to be engineered, and their functionality confirmed, inside cells and *in vivo*. A first example of an artificial organelle with functionality that can be switched on by changes in the cellular microenvironment has been very recently reported to preserve the architecture and functionality *in vivo*, in a zebra fish animal model [Figs. 7(f) and 7(g)].[79]

The advantages of this strategy, compared to alternative systems, include the protection of the active compound from proteolytic attack and a controlled functionality in the desired location (e.g., inside cells). Indeed, Palivan presented a promising example of this strategy, at BI 2016, with the simultaneous encapsulation of two different enzymes working in tandem inside a synthetic compartment based on PMOXA-PDMS-PMOXA combined with the insertion of membrane proteins into the compartment membrane in order to mediate the molecular permeability [Figs. 7(a)–7(c)]. In this way, an artificial organelle is generated that mimics the natural peroxisome process occurring inside native cells, whereby it efficiently detoxifies both superoxide radicals and H_2O_2 associated with oxidative stress and well known to be involved in pathologies ranging from cancer to HIV.[78] A step further is achieved if the *in situ* functionality is triggered by the presence of a stimulus, such as *p*H or glutathione. Such a triggered activity has thus far been successfully induced by chemically and genetically engineering the membrane proteins to serve as "protein gates" [Figs. 7(d) and 7(e)]. These protein gates open only in the presence of the desired stimulus and allow a molecular diffusion of substrates and products through the compartment membrane, which switches on the enzymatic reaction.[91,94] This has been further advanced by engineering stimuli-responsive artificial organelles based on simultaneous encapsulation of an enzyme involved in the cellular redox homeostasis and insertion of a genetically engineered channel porin to serve as a protein gate that triggers the enzymatic activity inside. As these artificial organelles, with triggered activity, preserve their integrity and remain functional *in vivo*, in the zebra fish animal model, they represent an important advancement toward the generation of multifunctional systems that will support the development of personalized medicine [Figs. 7(f) and 7(g)].[79]

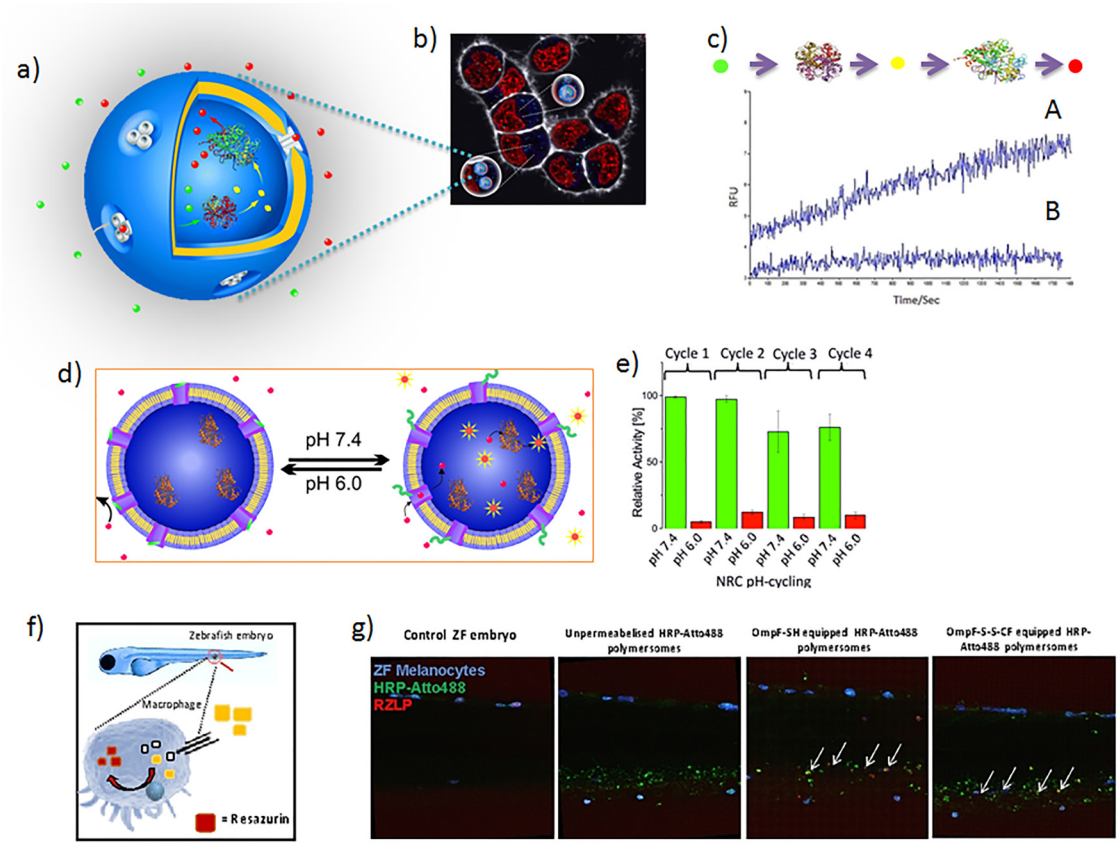

FIG. 7. (a) Schematic representation of artificial organelles based on an enzymatic cascade reaction taking place inside synthetic nanocompartments equipped with channel porins, which are (b) up-taken by cells. In (c) real-time reactive oxygen species detoxification kinetics of artificial peroxisomes, in (A) cells treated with pyocyanin, and (B) cells pre-treated with APs (8 h) followed by treatment with pyocyanin. (d) Schematic representation of artificial organelles equipped with biovalve functioning by reversible pore opening and closing inside the membrane of polymersomes to trigger an *in situ* reaction (left: closed state; right: open state). (e) Biovalve functionality when inserted into the membrane of horseradish peroxidase (HRP) loaded polymersomes (NRC) after changing the *p*H from 7.4 to 6.0, and adding Amplex UltraRed® and H_2O_2 in each cycle. Green: *p*H 7.4 and red: *p*H 6.0. The activities were corrected by taking the volume increase into account. (f) Schematic representation of artificial organelles injected in Zebra fish animal model. (g) Cellular uptake and intracellular activation of fluorescently labeled HRP-loaded polymersomes and fluorescently labeled HRP-loaded artificial organelles. Blue signal: Hoechst 33342 nucleus stain. Grey signal: CellMask Deep Red-Plasma membrane stain. Green signal: Atto-488 HRP. Red signal: resorufin-like product. Scale bar 20 μm. Adapted with permission from P. Tanner *et al.*, *Nano Lett.* **13**, 2875 (2013). Copyright 2013, American Chemical Society. Partially adapted from C. Edlinger *et al.*, *Nano Lett.* **17**, 5790 (2017). Copyright 2017, American Chemical Society. Also adapted in part from T. Einfalt *et al.*, *Nat. Commun.* **9**, 1127 (2018). Copyright 2018, Nature. See also Creative Commons Attribution 4.0 International License, https://creativecommons.org/licenses/by/4.0/.

A challenging aspect in the development of artificial organelles is the efficiency of co-encapsulation of different enzymes inside the compartment such that the resulting cascade reaction is supported in conditions similar to the natural organelles. In order to study the simultaneous presence of multiple enzymes inside the cavity of the nanocompartments, the enzymes can be fluorescently labeled and evaluated using fluorescence cross-correlation spectroscopy.[78] However, new bioanalytical methods that could enable the simultaneous detection and quantification of the enzymes inside the compartment are necessary for further development of the field, especially when there are two or more different types of enzymes, in which case information about their optimal stoichiometric ratio and the dependence of relative turnover rates on compartment size is crucial. Both membrane-loading and encapsulation efficiencies are parameters that are hard to control and often do not follow what one expects from stoichiometric estimates,[95] especially when taking correlations with nanoassembly size into consideration.[42]

Improved information regarding enzymatic heterogeneity, or the effect of being confined to nanometer range spaces, may, in future efforts, be obtained by combining functional (single enzyme) assays with information on structure and size that can be obtained with the nanopore-based and 2D flow nanometry concepts described in Secs. II and III, respectively. In addition, a key point in the functionality of artificial organelles is to preserve the activity of the enzymes inside the compartment, while allowing a rapid release of the desired products by a controlled permeability. The benefit of insertion of membrane proteins to render the compartment membrane permeable, when compared to porous membranes,[96] results from the possibility to control the number of biopores, or even to provide a selective permeability, depending on the specificity of the membrane protein, if required for specific reactions.[94] Therefore, in addition to the

study of the encapsulated enzymes, the development of bioanalytical tools to characterize the insertion of membrane proteins, as well as to characterize their structure and functionality inside the compartment membrane, is also expected to provide key insights and to aid in the optimization of artificial organelles. Similarly, advancements toward treating diseases with these novel concepts will also benefit from bioanalytical techniques that are capable of reporting on the fate of cellular function upon uptake, such as the ones described in Sec. V.

V. LIVE-CELL EXTRACTION FOR BIOMOLECULAR ANALYSES

As previously stated in this review, significant emphasis in contemporary drug discovery is placed on resolving the tissue and cellular targeting steps of nanoparticles designed for delivery of large molecular weight drugs, such as peptides, proteins, and oligonucleotides.[97] As many of these drugs act on the intracellular machinery, a means to investigate the molecular content of individual cells, to assess either the drug's intracellular level or the cell's molecular response upon exposure to the drug, has become increasingly crucial. Conventionally, the success of targeting, uptake, and cellular processing has been assessed by fluorescence microscopy, while cell sorting followed by cell lysis has been used to analyze the molecular content of cells.[97] The latter approach, however, is limited to the postmortem analysis of cells removed from their physiological context. Using fluidic force microscopy (FluidFM), Zambelli's and Vorholt's teams demonstrated, at the BI 2016 meeting, how an AFM-based approach can be used to accurately and gently drive a nanoscale probe through the cell membrane to extract tunable amounts of intracellular fluid from live cells and further that the withdrawn cell contents can be subsequently delivered to a variety of analytical techniques for further analysis.[7] In this section, we will discuss the potential of this new method, as well as a number of other single-cell approaches,[1] to assess the successful delivery of biologicals to living cells through the analysis of desired readouts.

Cell-based assays are widely used in biological and pharmaceutical research, providing a valuable intermediate between biochemical assays and animal studies. While cell-based assays are routinely performed on large cell populations, recent technological advances have now made it possible to scale molecular analyses down to the single-cell level, opening up a new era in biomedical research. Multicellular organisms comprise a variety of cell types and states, with each individual cell interacting with a specific microenvironment, and the biochemical processes within each cell being subject to stochastic fluctuations. In addition, cells are dynamic systems in which the molecular components change in time and space. The resulting cell-to-cell heterogeneity is imperceptible in classical studies performed on bulk cell populations, whereby the behaviors of numerous cells (typically 10^3–10^6 cells) are averaged. By contrast, SCA allow for the elucidation of cell-to-cell variations in transcripts, proteins, metabolites, and other analytes, thereby providing the means to study the origins and consequences of cellular heterogeneity and to investigate cellular state and function in development, health and disease without an averaging bias.

SCA have already provided invaluable insights into a variety of research fields, including developmental biology, neurobiology, immunology, stem cell, and cancer research. Notably, single-cell analyses have uncovered somatic mutations in the human genome,[98] resolved the cell type composition of various tissues, discovered previously unknown cell types,[99–101] and identified subsets of cells implicated in various diseases.[102–104] SCA undoubtedly holds great potential for drug development. They have already brought new insights into the molecular mechanisms of different diseases and have unveiled subsets of cells associated with drug resistance, providing valuable information for the development of novel therapeutic strategies.[105–107] In addition, SCA have enabled analysis of small molecule drugs and their metabolites in the cell interior, which is where most drugs are targeted.[108–111] More generally, SCA provides new means to evaluate whether a pharmaceutical compound reaches its action site in sufficient therapeutic concentration, and whether it engages with its molecular target and triggers the expected cellular response. At the same time, SCA can provide valuable information related to the heterogeneity in drug uptake and molecular response between different cell types.

While SCA is garnering an increasing interest from the biological and medical community, the field is still constrained by technical challenges. Mammalian cells typically have volumes of 1–5 pl, containing a broad diversity and a wide dynamic range of cellular analytes. For instance, a human cell contains more than 10 000 different proteins, 12 000 different transcripts, and 40 000 different metabolites, present at copy numbers spanning from one up to several million.[112–116] Molecular concentrations in cells are thus wide ranging but, considering the small volume of a cell, they typically lie between aM and mM.[117] While such concentrations do not directly challenge the limit of detection of current analytical instruments, tools to effectively handle volumes in the pl–fl range are lacking, and the cellular molecules are usually diluted in current workflows. The analysis of intracellular molecules is therefore an extremely difficult task that requires the implementation and further development of advanced bioanalytical technologies to enable the detection of low absolute amounts of analytes in complex mixtures. Moreover, physiological perturbations have to be minimal during the sampling in order to study unbiased molecular pools; metabolites, for instance, are highly sensitive to environmental changes and can react within seconds or even faster. Today, single-cell studies are therefore mostly focused on nucleic acids because they are relatively stable, and because amplification processes are available that make it possible to generate a sufficient concentration for further analysis.[118] Another limitation has been that, previously, SCA were, as schematically illustrated in Fig. 8, commonly

performed on dead (fixed or lysed) cells separated from their original tissue or cell culture. Although the dissociation, isolation, and lysis of individual cells can be achieved with high throughput, the workflow imposes important limitations: the contextual information of the analyzed cell is lost, non-negligible physiological perturbations afflict the cell and may influence its molecular profile, and the cell is ultimately sacrificed, preventing the collection of complementary data (e.g., cell behavior). Consequently, the use of SCA for investigating cell–cell communication, cell response to external stimuli, or any other spatially or temporally defined biological process has been limited.

A promising approach to address these limitations has emerged through breakthroughs in the nanotechnology field paired with the continuous technological development in bioanalytics. The alternative strategy, also conceptually illustrated in Fig. 8, consists of extracting and analyzing cellular biopsies rather than whole cells. Despite the decreased amount of molecules compared to the whole cell content, the approach is able to preserve cell viability and its physiological context, thus offering a means to broaden the potential of single-cell studies to include the spatially defined and temporal monitoring of individual cells. Several methods have been proposed to achieve such nondestructive biomolecular analyses, relying on the insertion of a minimally invasive nanoprobe into the cell interior to collect the intracellular molecules, followed by the transfer of the molecules to a suitable substrate to perform the downstream molecular analysis *ex situ*.

Nanoneedles, either arrayed on a substrate onto which the cells are cultured or used as individual probes manipulated externally with a micromanipulator, an AFM, or a magnetic field, represent one type of probe that enables sensing of the intracellular milieu.[119] Their small size provides minimal invasiveness, high spatial resolution, and high sensitivity upon insertion into single cells, thus making it possible to monitor intracellular biomolecules with minimal perturbations. The collection of endogenous molecules using nanoneedles has been achieved through molecular adsorption onto their surface, which can also be functionalized with antibodies, for example, to selectively capture a molecule of interest. Yet, adsorption of biomolecules onto the needle surface in a complex environment like the intracellular milieu is a phenomenon that can be neither monitored nor controlled. Insights into the adsorption kinetics, the local molecular concentration, or the surface coverage upon adsorption are unavailable, and the amount of molecules that can be collected is limited. By flowing the intracellular fluid into a hollow probe rather than adsorbing molecules on its surface, the limits, in terms of the quantity and variety of molecules that are sampled, can, in principle, be lifted. Singhal *et al.* immobilized single carbon nanotubes at the end of glass pipettes that were operated with a micromanipulator.[120] With an internal diameter of 50–200 nm, the endoscopes enabled the flow of attoliter volumes of fluids through the probe. The approach is promising for the study of the intracellular milieu with minimal perturbations; however, the molecular copy number contained in attoliter volumes still restricts the analytical options available. More recently, the Melosh group developed an approach based on a membrane with an array of hollow 150-nm-diameter nanostraws on top of which cells could be cultured.[121] Upon electroporation, intracellular proteins and mRNAs were shown to freely diffuse to microliter reservoirs filled with extraction buffer on the underside of the membrane. The extracted molecules were then transferred to the analyzer (ELISA and RT-qPCR) by a pipette. The approach allowed for multiple samplings of the same cell over time, with more than 95%

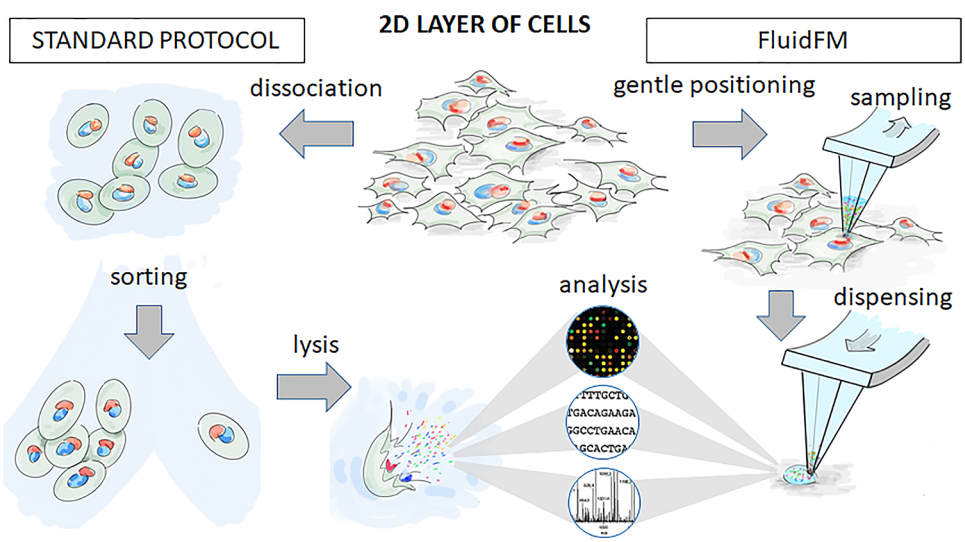

FIG. 8. Single-cell analysis. In the standard protocol (left), a cell culture or tissue sample is first dissociated to obtain a single cell suspension; the individual cells are then isolated and finally dissolved to make their molecular content accessible for the analysis. A problematic issue of this protocol is that the cells are decoupled from their environment and are no longer viable when analyzed. Alternatively, nanotechnologies such as the FluidFM (right) now make it possible to sample the cellular content while preserving cell viability and the intercellular interactions.

postextraction cell viability. In addition, while single nanoneedle probes are restricted to the serial sampling of individual cells, arrays of nanoneedles hold the potential for sampling multiple cells in parallel to enhance throughput in the future. Nonetheless, this promising approach is still limited in terms of the amounts of molecules that could be collected and analyzed. Passive diffusion for a few minutes enabled the collection of an estimated 70 fl, equating to ~1–7% of a whole cell's content. The method made it possible to follow temporal changes in the expression of a fluorescent reporter protein with single-cell resolution; however, qPCR assays to monitor targeted mRNAs required 15–20 cells, whereas enzymatic assays were performed on ~100 000 cells. With the potential to implement future advances in bioanalytics and to parallelize the sampling process, this approach offers great potential for enabling high throughput molecular analyses of single cells with minimal disturbance.

Another type of minimally invasive probe that has been proposed for sampling intracellular molecules is that of glass micro- and nanopipettes. Glass micropipettes were invented more than one hundred years ago to address and manipulate individual cells, mostly *in vitro* but also *in vivo* for electrophysiological experiments.[122] With respect to their bioanalytical application, the Masujima group lead the way with the "video-mass spectroscope,"[123] wherein a glass capillary is directly inserted into a living cell to withdraw its content and then positioned as a nanoelectrospray ionization (nano-ESI) tip in front of a mass spectrometer for the metabolic analysis of the sampled volume.[108,109,124–126] Nanospray microcapillaries with inner diameters of 1–5 μm, and operated with a micromanipulator, have enabled the collection of volumes in the attoliter to femtoliter range upon application of negative pressure.[123,127] Coupled to ultrasensitive nano-ESI mass spectrometry, the method succeeded in obtaining differential metabolite profiles from individual cells of different cell types and could even distinguish the metabolite content of different cell compartments, thereby identifying granule-specific molecules in mast cells. The method also enabled the measurement of two different drugs and their metabolites in the cytoplasm, nucleus, and vacuoles of human hepatocytes, offering great potential for the investigation of intracellular metabolism and cellular distribution of drugs. The advent of the scanning ionic current microscope provided a solution for the critical issue of the controlled approach onto the optically selected cell by taking advantage of the ionic current as a feedback signal.[128,129] Using scanning ion conductance microscopy (SICM)-operated glass nanopipettes with an aperture diameter of ~100 nm, Actis *et al.* succeeded in puncturing mammalian cells, collecting samples of cytoplasmic fluid by electrowetting, and analyzing both their mRNA and mitochondrial DNA content while assessing the cell viability after operation.[130] The collected volumes were estimated to be ~50 fl (~1–5% of the whole cell volume). The glass capillaries used in that study had dimensions approaching those of nanoneedles, thus reducing the cellular damage, that is, relatively common when using micropipettes, as evidenced by the cell viability postextraction of more than 70%. Nanopipette probes are easily produced from glass capillaries and represent a valuable alternative to nanoneedles.

AFM pyramidal tips have also been used for nondestructive sampling of intracellular molecules. Initially, developed for surface science investigations,[131] the AFM has also proved valuable in a number of biological studies, providing topographical as well as mechanical information at the molecular and cellular levels.[132,133] Osada *et al.* demonstrated how an AFM pyramidal tip can be inserted into the cell cytoplasm, maintained inside during the time necessary for the spontaneous physical adsorption of mRNAs onto the pyramid facets (~1 min), retracted out of the cell, and finally placed into a PCR tube.[134] By coating the AFM tip with successive conductive and insulating nanolayers of silicon/silica, an alternating bias can be applied to generate a dielectrophoretic effect that enables the attraction of nanosized objects. This phenomenon, sustained by a chemical functionalization of the AFM tip with appropriate primers, was successfully exploited by the Wickramasinghe group to increase the amount of mRNAs captured from the nucleus, which was then quantified by qPCR.[135] While dielectrophoresis enables an efficient mRNA enrichment, adsorption of the molecule of interest onto the probe surface remains, in most situations, not fully characterized like in the case of nanoneedles. Furthermore, methods based on the selective hybridization to chosen primers restrict the analysis to known RNA molecules.

By enabling the flow of intracellular fluid *into* an AFM cantilever instead of adsorbing selected molecules onto its tip, the recently invented fluidic force microscopy (FluidFM) provides an attractive alternative to conventional AFM. In particular, with microchanneled cantilevers, the AFM can act as a force-controlled pipette that merges the benefits of liquid exchange (from tens of femtoliters to several picoliters) with that of the gentle perturbation and nanoscopic precision of the AFM approach.[136] Guillaume-Gentil *et al.* have demonstrated, as presented at BI 2016, the collection of intracellular fluid, from individual cells, into hollow cantilevers upon application of an under-pressure tip inserted into either the cytoplasm or the nucleus [Figs. 9(a) and 9(b)]. The appropriate chemical functionalization of the walls of the microchannel to prevent unwanted molecular adsorption as well as to ensure cell viability after manipulation was comprehensively considered. The sampled volumes (from 100 fl to 8 pl) were analyzed with negative-stain transmission electron microscopy (cellular nanostructures), qPCR (mRNAs) and enzymatic assays (proteins),[7] and with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (metabolites) in an ensuing study[137] [Figs. 9(c)–9(e)]. The known channel dimensions and the possibility to observe the probe by optical microscopy enabled both quantification and tunability of the volumes of intracellular fluid extracted. This in turn allowed for the assessment of postextraction cell viability as a function of the volume of intracellular fluid removed, and it was found that more than 80% cell viability

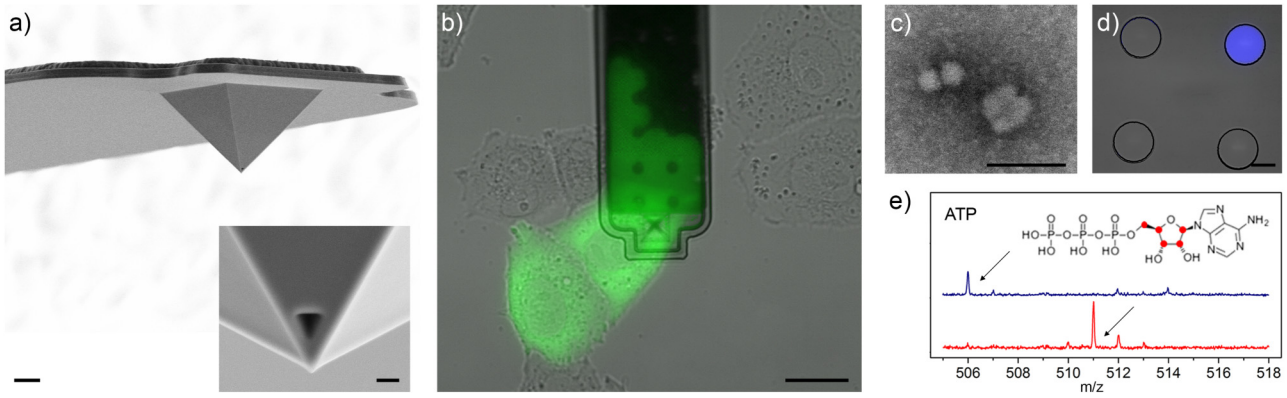

FIG. 9. Sampling and analysis of cellular biopsies by FluidFM. (a) Microchanneled FluidFM probe used for collecting cellular biopsies, featuring a 400 nm aperture (insert). Scale bars are 2 μm and 400 nm, respectively. (b) Harvesting of a cytoplasmic biopsy from HeLa cells expressing green fluorescent protein. Scale bar is 20 μm. (c) Negative-stain TEM image of extracted cytoplasmic molecules. Scale bar is 200 nm. (d) Fluorogenic assay for caspase-3 activity. The enzyme activity is detected in the upper right microwell following dispensing of a cytoplasmic extract, whereas the enzyme substrate is not converted in the three neighboring control wells without cell extracts. Scale bar is 20 μm. (e) Mass spectra of adenosine triphosphate (ATP) from cytoplasmic biopsies of HeLa cells fed with natural (blue) or ^{13}C-labelled (red) glucose. In part adapted with permission from O. Guillaume-Gentil *et al.*, *Anal. Chem.* **89**, 5017 (2017). Copyright 2017, American Chemical Society. In part adapted with permission from O. Guillaume-Gentil *et al.*, *Cell* **166**, 506 (2016). Copyright 2017, Elsevier.

was obtained after the removal of up to 0.6 pl of nucleoplasm and up to 4 pl of cytoplasm. While still ensuring cell viability, the extraction of relatively large volumes, under pressure control, facilitated downstream molecular analyses of the content extracted from single cells. The developed approach also benefits from the decisive force feedback that enables the careful dispensing of the extracted samples onto the matching analyzer substrate (grids, wells, and liquid droplets) without crashing the probe.

The emerging approaches based on cellular biopsies presented herein (and summarized in Table II) broaden the potential of single-cell analytics, thereby offering attractive opportunities for spatially defined and temporal molecular analyses of living cells in their physiological context. A major challenge facing the application of these approaches in biomedicine will be the expansion of the analytical capabilities for going beyond proof-of-principle studies towards comprehensive omics analysis. Although cellular biopsies contain smaller absolute number of analytes, and in smaller volumes compared to entire cells, the massive efforts currently invested to advance whole cell analysis will also benefit that of cellular biopsies. Future progress in the field will strongly depend on the development and implementation of cutting-edge bioanalytical platforms to further lower the current limits of detection and to achieve more comprehensive analyses of the diverse cellular compounds. While such

TABLE II. Summary of single-cell biopsy technologies.

Nanoneedles	Aperture diameter	Extraction mechanism	Cell compartment	Volumes collected	Cell viability	Single-cell molecules analyzed
Nanoendoscopes	50–200 nm	Diffusion	Cytoplasm	al	100%	—
Nanostraws[a]	150 nm	Diffusion	Cytoplasm	70 fl	>95%	Proteins (RFP)
Glass pipettes						
Nano-ESI tip	1–5 μm	Pressure-controlled suction	Cytoplasm Nucleus Vacuoles	al–pl	n.a.	Metabolites
Nanopipette	100 nm	Electrowetting	Cytoplasm Mitochondria	50 fl	>70%	mRNAs miDNAs
AFM cantilevers						
AFM	—	adsorption	Cytoplasm Nucleus	—	n.a.	mRNAs
DENT-AFM[b]	—	Dielectrophoresis and adsorption	Cytoplasm Nucleus	—	n.a.	mRNAs
FluidFM	400 nm	Pressure-controlled suction	Cytoplasm Nucleus	100 fl–8 pl	>80%	mRNAs Proteins (enzymes) Metabolites

[a]For single cells, arrays of 10 000 nanostraws in a 100 μm × 100 μm substrate.
[b]Dielectrophoretic nanotweezer-AFM.

technological improvements are expected in the near future, the different strategies reported herein could already be applied to investigate biological processes and to assist in the development of biopharmaceuticals. For example, the measurement of transcripts, enzyme activities, or metabolites is becoming feasible. Furthermore, breakthroughs in single-cell bioanalytics, as reported here, could offer critical insights regarding cellular response after exposure to a biopharmaceutical as well as for the direct measurement of a biological (e.g., therapeutic oligonucleotides) in the cell interior. With the exception of nucleic acids, it thus far remains a challenge to obtain the sensitivities needed to analyze all types of biomolecules found in low abundance, and, in particular, to observe changes in protein concentration. However, the advancement in single protein analytics reported in this paper provides reason to be very optimistic regarding future possibilities to evaluate both the efficacy of different nanocarriers to deliver the drug into the cell and its potency to, for example, produce proteins or to influence their expression levels.

VI. OUTLOOK

It is obvious that there remains much to be gained from a continued effort to mimic the elegant solutions that have evolved in nature, with respect not only to virus-, exosome-, and organelle-inspired drug-delivery vehicles but also bioanalytical aspects, as here illustrated with methods that make use of the two-dimensional fluidity of cell-membrane mimics for protein and nanoparticle characterization. Progress in these directions will depend strongly on enduring collaborative multidisciplinary enterprises that should, from the start, match the needs in both academic and clinical settings with cutting-edge method development. To understand how, for example, nanoparticle size correlates with charge, shape, or ligand density, and to understand how such correlations influence biological function, it is critical that individual nanoparticles can also be sorted according to certain predefined fingerprints. In analogy with fluorescence activated cell sorting (FACS) of individual cells, such techniques should ideally characterize particles in solution and be sufficiently rapid to provide a decision for sorting. Despite recent advancements in the field, nanoparticle sorting using conventional methods such as field flow fractionation[138,139] and flow cytometry[140] (which can today offer sensitivities on the single nanoparticle level approaching that of NTA[141]) are so far not compatible with sorting on the single nanoparticle level. An important contribution that might open up for this challenging goal was recently demonstrated using so-called deterministic lateral displacement pillar arrays.[142] Thanks to developments in integrated nanofluidic chip designs, this concept offers visualization and separation of biological nanoparticles with dimensions down to a few tens of nanometers. Further, the planar design of the chip means that the nanoparticle could remain in optical focus throughout the separation step, which suggests that correlation with molecular content could be feasible. Both the 2D flow nanometry and the nanopore concepts could, in principle, also be combined with a separation step since microscopic inspection and real-time analysis of moving nanoparticles can be used to trigger downstream sorting such that a sorting decision could be made based on the results from this analysis. To be practical, however, such an approach would have to operate in high throughput and would require increased temporal resolution of the recordings while maintaining a high signal-to-noise ratio, improved chip fabrication, advanced computational algorithms, the ability to combine analysis with fast-switching fluidics, and to have this all adapted to an array format supporting hundreds or thousands of particles or pores. While this collection of required improvements may sound like a daunting list, rapid advances in micro- and nanofabrication,[143] as well as in the design of integrated circuits,[144] have already accomplished some of these goals and we are optimistic that significant improvements will be made in the years to come. Essentially such advancement would be analogous to FACS,[145] with the distinction, at least if operated in label-free mode, that it would be capable of separating individual molecules or nanoparticles, rather than single cells, and that the signal for sorting would result directly from particle properties rather than from a fluorescent label. It is in this context relevant to recall that progress has been made in handling tiny amounts of liquids, as well as the rapid development of tools compatible with advanced single-cell manipulation (see Sec. V), thus promising that significant advances in understanding cellular uptake and functional response can be expected even before the throughput of such nanoparticle sorting devices has reached the same level as state-of-the-art FACS. Similarly, a functional understanding of the type of artificial organelles discussed in Sec. IV would also benefit from this type of single-cell investigation.

An additional aspect of key importance for single-cell and single-nanoparticle analytics is the role of the surface modifications required for the techniques to function optimally, as all of them stand and fall with how they succeed in producing the right interface for the investigations. The fluid coating at the interface utilized for both the 2D flow nanometry and nanopore concepts is composed of a supported lipid bilayer made of zwitterionic phospholipids, which offer several critically important properties. For instance, its chemical and physical surface properties have been evolutionarily selected to minimize interactions with the majority of soluble biomolecules. In fact, the nonadhesive properties of lipid coatings were found to be superior to any previously reported coatings in nanopores including those from self-assembled monolayers of PEGylated alkane thiols on gold.[54] Circumventing such nonspecific adsorption to the pore walls was the critical prerequisite for a quantitative analysis of translational and rotational dynamics since adhesive interactions would lead to artifacts in the determined rotational diffusion coefficient, dipole moment, and charge (see Sec. III). Similarly, too high an adhesion would render nanoparticles immobile under 2D flow nanometry analysis, thus excluding size

determination (see Sec. II). One additional benefit of coating nanopore walls with an SLB is that the surface charge of the coating can be tuned by the choice of lipid headgroups. This capability makes it possible to approach a surface potential close to zero and thereby to circumvent electroosmotic flow in the nanopore in the presence of an electric field.[146] Since convectional flow through the nanoscopic constriction of the nanopore is negligible in the absence of large pressure gradients, the translational motion of proteins is purely diffusive and electrophoretic. As an added benefit, bilayer coatings make it possible to incorporate lipid anchors with affinity towards specific analytes. For instance, lipids displaying biotin groups on their headgroups made it possible to capture, concentrate, and analyze biotin-binding proteins on the surface, as demonstrated for both nanopores[54] and flow nanometry.[147] The well-established streptavidin–biotin coupling strategy can also be easily extended into sandwich configurations, in which lipid-anchored streptavidin binds biotinylated targets of choice, such as antibodies, protein A/G, enzymes, and oligonucleotides. Further, Höök's and Boxer's groups have pioneered an alternative strategy, making use of lipid modified DNA that self inserts into lipid membranes.[148,149] This enables sequence specific tethering of DNA-modified nanoparticles that become mobile at the lipid membrane interface, given that the number of DNA tethers is less than around 10–20.[150] These strategies, however, put some limitations on the nature of nanoparticles that can be analyzed since they require some kind of biomolecular functionalization. However, Cho *et al*. have shown that electrostatic attraction can also be used to couple lipid vesicles in a mobile state on supported membranes,[151] suggesting that it will be possible to bind and analyze both polymeric and inorganic nanoparticles by varying the charge of the membrane via the lipid composition. Further, supported membranes are often used to avoid nonspecific biomolecular adsorption to the walls of nanoscale extraction tools, like the FluidFM probe, as they have been shown to be superior to many other coatings when handling oligonucleotides in nanochannels.[152] However, if there is no explicit need for a fluid coating, there are several alternatives, such as the protein-resistant polymer poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG, Surface Solutions GmbH). The nonfouling properties of PLL-g-PEG coatings are greatly improved using higher temperatures during the polymer adsorption on the surface, thereby yielding higher grafting densities,[153] which have been demonstrated very efficiently as a means to reduce nonspecific adoption to the walls of nanoscale cellular extraction tools.[133] PLL-g-PEG can also be combined with biotinylated PLL-g-PEG,[154] offering the interesting opportunity to use the inner walls of extraction probes for selective identification of predefined intracellular target molecules. It is from these examples clear that further advances in surface modification strategies will continue to play a critical role in the development of both bioanalytical tools and drug-delivery vehicles, and virtually all aspects of biomedical devices and materials.

It is also important to stress that it is not only multidisciplinary efforts that are needed to bridge existing knowledge gaps of the type mentioned above, as there are several additional barriers that must be overcome to successfully translate, for instance, state-of-the-art analytical tools emerging from physical sciences in engineering settings, to biological, medical, and pharmaceutical settings. In the case of the nanopore technology concept presented in Sec. III, it has indeed been made broadly available, thanks to commercialization efforts with DNA sequencing in mind.[56] However, if the potential market size is not as large, as in the case of genetic screening, the investment required for constructing and successfully launching a sufficiently robust and user friendly tool is often considered too high a barrier. The fluidic force microscopy concept (FluidFM) presented in Sec. V is one example of a methodology that, despite this challenge, has been made available to a broad research community, and it is indeed our hope and belief that the method could potentially be an important complement to existing single-cell manipulation tools. However, in addition to efficient funding schemes, there are many other barriers that need to be overcome in making academic inventions broadly available, which together form what is known as the "valley of death" for small start-up companies. For a general discussion on this topic, we refer the interested reader to the online interviews that were made with selected speakers at the BI 2016 meeting, and, in particular, Interview 5: Hurdles impeding progress in the field and in the translation of basic discoveries.[155]

As mentioned in the interview with Dr. Textor (see the Appendix), a particularly challenging area that requires further concerted and interdisciplinary research is in the study and comparison of single-cell responses in different culture systems from *in vitro* studies of cells on surfaces to co-cultured cells in 3D tissue-model environments that more closely resemble *in vivo*. Such 3D model systems were described during the BI conference, including work by Dr. M. Lutolf and Dr. B. Rothen-Rutishauser, wherein methods for creating 3D, highly controlled, microenvironments, and utilizing dynamic materials were presented with the perspective of both studying and manipulating specific disease tissues both in the laboratory and in the body.[156–158] We can further foresee that tests of the type of drug-delivery vehicles that have been highlighted in this review will benefit importantly from 3D organoids of this nature. Further, the spatial flexibility of the FluidFM instrument could be, in the future, further developed into an excellent tool to examine individual live cells grown in such models. One can also envision that the very poorly understood, to date, but critical endosomal-escape step that needs to be overcome for next generation nucleotide drugs to reach optimal efficiency[3] could be characterized in detail by using single-cell extraction to isolate endosomes at different stages of the internalization process, and to use nanoparticle characterization tools of the type reviewed here to gain information about their molecular composition and structure at different stages in the process.

By having addressed these and related questions in the context of evaluating progress in the field of bioanalytical advances, we hope to have stimulated further research and efforts to cross disciplines and bridge the gap between research in engineering settings, clinical applications, as well as small start-up companies and well-established biotech and pharmaceutical industries.

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APPENDIX

Interview with Professor Marcus Textor, Chair of the inaugural Biointerfaces International Conference, Zurich, August 2016.

HMG: What prompted the genesis of the Biointerface International conference and its unique format, which included a day dedicated to translation of innovation to clinic and industry?

MT: Our starting point was a small team discussing the potential need for a conference format that would distinguish itself from the many types of conferences in the life sciences field including the smaller conferences focused on a specific scientific subject such as the Gordon Conferences and the much larger professional society type conferences, like the MRS and ACS. We identified a need for a medium sized, international conference with the aim of providing high-level scientific talks, covering the most innovative and expanding topics across the very diverse fields of relevance to the topic of biological surfaces and interfaces, and at the same time making it attractive to attendees from academia, industry and clinics interested in the translation of scientific discovery to markets and clinics. The conclusion was to go for a 3-day conference that included (1) a three-day single session of high-level science spanning from bioinspired materials and bioanalytical tools to 3D cell cultures and regenerative medicine presented by a mix of internationally renowned speakers, early-career scientists and speakers from industry; (2) a one-day parallel session on translation including a "Science to Market" component covering pitching science to markets, start-ups and corporate ventures, followed by a "Science to Clinics" component addressing some of the difficulties in translation; and (3) a poster session to stimulate discussions among groups. The hope is that the unique format of this medium-sized conference, combined with the intention to identify new communities and latest developments in the field every two years, will ensure that we continue to attract future participants from the international community in academia, industry and clinics, across all career levels.

HMG: During the conference, and judging from how you have seen this field develop, did you identify any key areas as lacking for the future progress in this interdisciplinary field?

MT: Yes, there are many hurdles impeding progress in the field and particularly in terms of translation of basic discoveries to clinical and industrial applications. For instance, David Mooney, a professor from Harvard University who is a leading expert in mechano-transduction and new medical therapies, identified a need for adjustments in the infrastructure and funding structure of translational research. The current funding structure was said to be lacking the move to first-in-man studies or to take innovation through the "last mile" in translation, also known as the "Valley of Death." Funding of, and encouragement of, even wider interdisciplinary approaches and teams to create biologically functional and translatable constructs such as dynamic functional systems and organoid structures, for both diagnostic applications, e.g., drug screening, toxicity, disease monitoring, as well as for applications in targeted drug delivery and regenerative medicine, is needed. Validation as to whether the resulting data truly represents human biology or whole body biology remains to be shown, however, and this pursuit will benefit from quantitative bioanalytical tools that can, for example, enable the study and comparison of single-cell responses in different culture systems from 2D to 3D. Furthermore, it was noted that future progress in the wider field of biomaterials would depend on the development and accessibility of the increasingly sophisticated technology required to probe the many relevant biointerfaces including those found in both biological and synthetic nanoparticles.

HMG: The notion of bioinspired solutions and biomimicry came up frequently during the conference; what are your thoughts on the current state of the art and future directions when taking nature as our guide?

MT: For me this is one of the most exciting and promising fields in materials science. It's an unlimited source of inspiration. Bioinspiration has resulted in a number of functional materials, interfaces and devices, when scientists have either chosen concepts wherein natural materials or components are used, e.g., self-assembled systems such as multifunctional lipid vesicles, biological nanoparticles, or where artificial systems are used to mimic, as closely as possible, native mechanisms or functions. With the latter, biomimicry systems or devices can in principle be based on a great variety of designed materials, such as stable supported polymeric membranes for surface-sensitive sensing and polymeric vesicles as nanocontainers. Native material constructs have the advantage of exploiting very efficient natural pathways, often difficult to mimic by artificial materials, and require, in general, reduced regulatory effort. An additional very important objective for this conference was to show the latest concepts and achievements in this competitive field towards applications in bioanalytics and clinical diagnostics with unprecedented sensitivity (e.g., single protein analysis,

single nanoparticle characterization) as well as towards advanced solutions for drug delivery. A common challenge, as specifically addressed by hosting a translation session, will be a means to successfully transfer these new technologies towards clinical evaluation and practice, and their validation as reliable tools.

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